Further Characterization of *Achromobacter nematophilus* from American and Soviet Populations of the Nematode *Neoaplectana carpocapsae* Weiser

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Cultural and biochemical studies showed no significant differences between a bacterium isolated from the nematode *Neoaplectana carpocapsae* in the Soviet Union and the bacterium, originally described as *Achromobacter nematophilus*, isolated from the DD-136 strain of *N. carpocapsae* in the United States. Further characterization of *A. nematophilus* is presented, but its reallocation to a more suitable genus should await the reorganization of the families *Achromobacteraceae* and *Enterobacteriaceae* now in progress.

The bacterium *Achromobacter nematophilus* was originally isolated from the intestine of the DD-136 strain of the nematode *Neoaplectana carpocapsae* Weiser (6-8). Further studies demonstrated the importance of *A. nematophilus* in the development and reproduction of this entomogeneous nematode (9).

It was of interest to know if other neoaplectanid nematodes or geographical populations of the same species had formed associations with identical or related bacteria. Besides being of interest to nematode taxonomists, who could characterize each neoaplectanid nematode by its specific associated bacterium, such findings would also be helpful in understanding the evolutionary development of this genus of nematodes.

By using the technique described by Poinar (6), a specific bacterium was isolated from a new strain of *N. carpocapsae* Weiser obtained from the Soviet Union, and detailed studies were made comparing this bacterium with the original *A. nematophilus* isolated from the DD-136 strain of this nematode in the United States. The results of these studies, along with a supplementary description of *A. nematophilus*, are presented here.

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MATERIALS AND METHODS

A freeze-dried culture of *A. nematophilus* Poinar and Thomas (8) obtained directly from the DD-136 strain of *N. carpocapsae* Weiser at the University of California was used and will be referred to as the DD-136 strain of *A. nematophilus*. From the Soviet Union, a similar bacterium was isolated from a neoaplectanid nematode collected from the body cavity of an elaterid beetle, *Agriotes lineatus* L., near Leningrad. Although originally thought to be a different nematode (11), breeding tests showed that the Soviet neoaplectanid could interbreed with the DD-136 strain of *N. carpocapsae* and that they belonged to the same species. The Soviet nematode has been referred to as the "agrios strain" of *N. carpocapsae* Weiser (Poinar and Veremtschuk, *Zoolog. Z.*, in press), and the bacterial isolate from this nematode will be referred to as the "agrios strain."

Bacterial colonies from both sources were incubated at room temperature (24 C) for determining their biological and cultural characteristics. Measurements of cells were made with 24-hr peptone-water (P-HOH) shake cultures, consisting of 1% peptone and 0.5% NaCl. Colony characteristics were made on 24-hr nutrient agar (NA) cultures and on 1- to 7-day Tergitol-7 agar cultures with TTC (T-7 + TTC). Tergitol-7 agar (3) consists of 5 g of peptone, 3 g of yeast extract, 10 g of lactose, 15 g of agar, 0.1 ml of Tergitol-7, and 0.025 g of bromothymol blue per liter. The TTC (triphenyltetrazolium chloride) is added to the Tergitol-7 agar at the rate of 40 mg/liter. *A. nematophilus* is unique in forming blue colonies on a light-blue background on this medium, and this reaction can be used as a diagnostic character for this

bacterium. Motility broth consisted of 18.5 g of Brain Heart Infusion plus 5.0 g of glucose per liter of water and was incubated for 18 hr. Leifson's method (5) was used to determine flagellation. Potato slants were prepared by the method of Collins (1). Catalase activity was determined by flooding colonies on NA with 10% hydrogen peroxide, and oxidase activity was checked by flooding colonies on NA with 1% aqueous tetramethyl-*p*-phenylenediaminedihydrochloride plus 0.1% ascorbic acid. Cytochrome oxidase activity was tested by using Schaefer's method (10). Methods involving the use of the following tests were obtained from the 9th edition of the Difco Manual (3) and Difco Supplementary Literature (4): AC medium, litmus milk, Leoffler's blood serum, gelatin liquefaction, MacConkey agar, Triple Sugar Iron agar (TSI), lysine decarboxylase, Simmon's citrate, phenylalanine deaminase, indole production, nitrate reduction, methyl red and Voges-Proskauer tests. Tests utilizing Hugh and Leifson's oxidation-fermentation medium and arginine dihydrolase and lysine and ornithine decarboxylases, after Møllers method, were described by Cowan and Steel (2). Anaerobic cultures were grown in a Torbal model AJ-2 jar filled with hydrogen. Measurements of individual cells were made by examining 50 randomly selected rods and spheres, respectively, of both strains.

Fermentation studies were conducted with 1% of each test sugar in P-HOH adjusted to pH 7.0 with NaOH. Two indicators were used: bromothymol blue, sensitive to slight acid changes in pH; and bromocresol purple, sensitive to stronger acidity. By using a double check on fermentative activity, reversals in the pH of weakly buffered solutions could be detected. These tests, with appropriate controls, were checked every 24 hr for 7 days.

Growth requirements were studied in 24-hr P-HOH cultures, and growth was measured by comparing absorption of the test culture to that of a sterile blank made by using a Bausch & Lomb Spectronic 20 colorimeter-spectrophotometer, at a wavelength of 600 nm. Temperature tolerance was studied in P-HOH at pH 7.0, and pH tolerance was conducted at room temperature in shake cultures of P-HOH. Sodium chloride tolerance was also conducted in P-HOH shake cultures at room temperature.

RESULTS

Results of the cultural and biological studies are summarized in Table 1. Bacteria from both sources were almost identical under the conditions of these experiments. The cells were large, thick, motile, gram-negative, nonsporeforming rods, occurring singly, in pairs, and rarely forming longer chains. In older cultures, spherical cells were formed, resulting from a disintegration of the cell wall.

On NA, the colonies were about 1 mm in diameter, greyish-white, convex, circular, with slightly irregular margins, and exhibiting a somewhat rough, granular appearance.

On T-7 + TTC medium, young colonies (less

than 30 hr) may be grey, but then rapidly turn blue, becoming darker with age and developing deep red to maroon centers. The reaction is alkaline and the background turns blue; with 3- to 5-day-old cultures, the bromothymol blue becomes absorbed by the cells, leaving clear zones in the agar around the colonies.

The average lengths of bacterial cells from both strains were similar and ranged from about 1 to 6 μ m. Both strains had peritrichous flagellation. Neither strain produced pigment on NA, in P-HOH, or on potato; however, both produced a brown pigment on Leoffler's blood serum. Both strains were aerobic in AC medium, and reached depths of 12 mm and 18 mm, respectively. Curd in litmus milk was produced by both strains, which also were able to liquefy gelatin. The strains were negative for the following enzymes: catalase, oxidase, cytochrome oxidase, arginine dihydrolase, lysine and ornithine decarboxylases, and phenylalanine deaminase. Neither strain utilized citrate, produced indole, or reduced nitrate to nitrite. Both were negative for the methyl red and Voges-Proskauer tests. Hugh and Leifson's O-F medium showed both strains to be fermentative in their metabolism of glucose. Some growth of both strains was obtained in the Torbal anaerobic jar after 4 days on NA.

On the basis of these results, we will expand on the original characteristics presented for *A. nematophilus* (8). In the present studies, good growth was obtained on MacConkey agar at room temperature in 24 hr, whereas, in the original description, little or no growth was reported at 37 C for 3 to 7 days. Again, good growth on potato was obtained at room temperature in the present studies in contrast to the original report of no growth at 30 C. An acid reaction was reported on TSI at 20 C in the original description, but the same test in the present investigation at room temperature was neutral. These differences point out the importance of running tests at optimum temperature for the organisms.

All of the 19 carbohydrates tested in the fermentation studies were weakly positive. Positive results were obtained by using weakly buffered solutions with adonitol, dulcitol, lactose, mannitol, rhamnose, salicin, sorbose, and sucrose, all of which were recorded as negative in the original description of *A. nematophilus*. Some of the sugars giving positive results were tested for the first time. These include esculin, melezitose, melibiose, raffinose, and saccharose.

The results of the studies on tolerance to environmental conditions are recorded in Table 2. The temperature tolerance study showed

TABLE 1. *Cultural and biological characteristics of the DD-136 and Agriotos strains of Achromobacter nematophilus isolated from separate populations of Neoplectana carpocapsae*

Test (room temp = 24 C)	Bacterial strains ^a	
	DD-136 strain	Agriotos strain
Cell size (P-HOH; 24 hr)		
Rods (avg length × width)	5.4 × 1.1 μm	5.0 × 1.1 μm
Spheres (avg diameter)	2.5 μm	2.4 μm
Motility broth	+	+
Flagellation	Peritrichous	Peritrichous
Chromogenesis (NA and P-HOH)	—	—
Aerobic (AC Medium)	+	+
Litmus milk (7 days)	+	+
Reduction peptonization	+	+
Curd	+	+
Loeffler's blood serum (5 days)		
Proteolysis	+	+
Pigment	Light brown	Light brown
Gelatin liquefaction (7 days)	+	+
MacConkey Agar (24 hr)	+w	+
Triple Sugar Iron agar	NC, NC, —	NC, NC, —
H ₂ S (TSI) (24 hr)	—	—
Potato slant (3 days)		
Growth	+	+
Pigment	—	—
Catalase	—	—
Oxidase	—	—
Cytochrome oxidase	—	—
Lysine decarboxylase	—	—
Arginine dihydrolase	—	—
Ornithine decarboxylase	—	—
Simmons citrate	—	—
Phenylalanine deaminase	—	—
Indole production	—	—
Nitrate reduction	—	—
Methyl red	—	—
Voges-Proskauer	—	—
Hugh and Leifson's oxidation-fermentation medium		
Open tube	+	+
Closed tube	+	+
Anaerobic NA culture	+w	+w
Fermentations: (24 hr)	Bt.B.:B.C.P.	Bt.B.:B.C.P.
Adonitol	+w:—	+:—
Arabinose	+:—	+:—
Glucose	++:	++:
Dulcitol	+w:+w	+w:+w
Esculin	—: +w	—: +w
Fructose	+w:+w	+w:+w
Inositol	+w:+w	+w:+w

TABLE 1. *Cultural and biological characteristics of the DD-136 and Agriotos strains of Achromobacter nematophilus isolated from separate populations of Neoplectana carpocapsae (Con't.)*

Fermentations: (24 hr)	Bt.B.:B.C.P.	Bt.B.:B.C.P.
Lactose	+w:+w	+w:+w
Mannitol	+w:+w	+w:+w
Melezitose	+w:+w	+w:+w
Melibiose	+w:+w	+w:+w
Raffinose	+w:+w	+w:+w
Rhamnose	+w:+w	+w:+w
Saccharose	+w:+w	+w:+w
Salicin	+w:+w	+w:+w
Sorbose	+w:+w	+w:+w
Sucrose	+w:+w	+w:+w
Trehalose	+:+w	+:+w
Xylose	+w:+w	+w:+w

^a Symbols and abbreviations: —, negative; +, positive; +w, weakly positive; NC, no change; Bt.B., Bromothymol blue indicator; B.C.P., bromocresol purple indicator.

TABLE 2. *Growth studies of the Agriotos and DD-136 strains of Achromobacter nematophilus under varying conditions of temperature, pH, and salt concentration*

Conditions studied		Absorption of bacterial strains ^a		
		DD-136 strain	Agriotos strain	Control
<i>Temp tolerance</i>	<i>C</i>			
pH 7	10	1	1	0
0.5% NaCl	24	6	5	0
	30	14	10	0
	37	1	1	0
	42	0	0	0
<i>pH tolerance</i>	<i>pH</i>			
24 C	5.0	0	0	0
0.5% NaCl	6.0	0	0	0
	7.1	69	4	0
	8.2	57	2	0
	9.0	31	3	0
	10.0	0	0	0
<i>Sodium chloride tolerance</i>	<i>Per cent concn of NaCl</i>			
24 C	0.0	0	0	0
pH 7	0.5	90	67	0
	1.0	82	62	0
	3.0	1	1	0
	4.0	0	1	0
	5.0	0	1	0

^a Growth expressed as per cent absorbance in 24 hr. P-HOH culture, measured in a Bausch & Lomb Spectronic 20 colorimeter-spectrophotometer, at a wavelength of 600 nm. A reading of 1 on the Spectronic 20 is considered equivalent to 0 for the purposes of this study.

that both strains reached a growth peak at 30 C.

The results of the pH study showed best growth for both strains at pH 7.1. No growth occurred below pH 6.0 nor above pH 10.0.

Both strains showed the same tolerance for sodium chloride, reaching a growth peak at 0.5% and showing good growth at 1.0%. There was little or no growth above 3.0%.

DISCUSSION

These studies show no significant differences between the bacterium isolated from a strain of *N. carpocapsae* in the Soviet Union and *A. nematophilus* isolated from the DD-136 strain of *N. carpocapsae* from the United States and, for all taxonomic and biological purposes, they may be considered the same species.

It is possible that the members of the *Achromobacteraceae* will be characterized as being strict aerobes (A. J. Holding, *personal communication*), and, if this is the case, our tests showing that *A. nematophilus* is capable of anaerobic development may remove this species from the genus *Achromobacter*. Thus, *A. nematophilus* may eventually be shifted into the family *Enterobacteriaceae*, although the negative catalase reaction might restrict its placement somewhat.

Serological tests comparing antisera of *A. nematophilus* with various bacteria from the families *Achromobacteraceae* and *Enterobacteriaceae* are now in progress and will be reported at a later date.

Since the diagnostic characters of many of the existing genera in *Achromobacteraceae* and *Enterobacteriaceae* will most likely be changed with the new edition of *Bergey's Manual*, assignment of *A. nematophilus* to another genus would be premature at this time.

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